

**HUMAN FOLLICLE STIMULATING HORMONE (hFSH) AND  
THYROXINE (T<sub>4</sub>) IN SURVIVAL MAINTENANCE AND IN VITRO  
GROWTH PROMOTION OF CAPRINE PREANTRAL FOLLICLES**

***HORMÔNIO FOLÍCULO ESTIMULANTE HUMANO (hFSH) E TIROXINA (T<sub>4</sub>)  
NA MANUTENÇÃO DA SOBREVIVÊNCIA E PROMOÇÃO DO  
CRESCIMENTO IN VITRO DE FOLÍCULOS PRÉ-ANTRAIS CAPRINOS***

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**Abstract**

The aim of this study was to investigate the interaction of human FSH (10ng/ml) with T<sub>4</sub> (20ng/mL) on survival, activation and growth of preantral follicles cultured *in vitro* for 28 days. Fragments of non-cultured and cultured ovarian tissue were processed for classic histology and transmission electron microscopy. The results showed a reduction in the survival rate in all the media tested (one to 28 days) when compared to the fresh control. However the treatment with T<sub>4</sub>/hFSH for seven days of culture maintained the rate similar to the control. The media tested by one and 28 days reduced the percentage of primordial follicles in all periods of culture. However, T<sub>4</sub>/hFSH on day one of culture remained similar to the fresh control. None of the media were able to keep the percentage of the developing follicles. It was observed that the follicular diameter in the medium with T<sub>4</sub>/hFSH remained similar to the fresh control. The ultrastructural analysis confirmed the integrity of follicles cultured for seven days in a medium supplemented with T<sub>4</sub>/hFSH. In conclusion, the medium with T<sub>4</sub>/hFSH is able to maintain the survival, promote the activation, and the ultrastructural integrity of caprine preantral follicles for until seven days.

**Keywords:** Activation; caprine; hFSH; preantral follicles; T<sub>4</sub>.

**Resumo**

O objetivo deste estudo foi investigar a interação do FSH humano (10 ng/mL) com T<sub>4</sub> (20 ng/mL) na sobrevivência, ativação e crescimento de folículos pré-antrais cultivados *in vitro*, por um período de longa duração (28 dias). Fragmentos de tecido ovariano não cultivado e cultivados foram

processados para histologia clássica e microscopia eletrônica de transmissão. Os resultados mostraram uma redução na taxa de sobrevivência em todos os meios testados (um a 28 dias) quando comparado ao controle fresco. Entretanto, o tratamento com T<sub>4</sub>/hFSH por sete dias de cultivo resultou em taxa semelhante ao controle. Os meios testados por 28 dias reduziram o percentual de folículos primordiais em todos os períodos de cultivo. Contudo, T<sub>4</sub>/hFSH no dia um de cultivo manteve-se semelhante ao controle fresco. Nenhum dos meios foi capaz de manter o percentual dos folículos em desenvolvimento. Observou-se que o diâmetro folicular cultivado no meio com T<sub>4</sub>/hFSH manteve-se semelhante ao controle fresco. As análises ultraestruturais confirmaram a integridade de folículos cultivados por sete dias em meio suplementado com T<sub>4</sub>/hFSH. Em conclusão, o meio com T<sub>4</sub>/hFSH é capaz de manter a sobrevivência, promover a ativação e a integridade ultraestrutural de folículos pré-antrais caprinos, por até sete dias.

**Palavras-Chave:** Ativação; caprino; folículos pré-antrais; hFSH; T<sub>4</sub>.

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## Introduction

The limitation in the supply of fertilizable mature oocytes represents an important obstacle to increase the success of assisted reproduction techniques. Accordingly, the use of *in vitro* oocytes of cultured and matured follicles is an alternative to the improvement of these techniques<sup>(1)</sup>. Over the last decades, some advances have been achieved during *in vitro* culture of preantral follicles; however the rate of embryo production is still low in domestic animals<sup>(2, 3)</sup>. This low efficiency can be caused by the media composition used during the culture period. These are unable to withstand the constant evolution of the developing follicle needs<sup>(4)</sup>.

During the reproductive life of the female, a large number of primordial follicles are stimulated to grow, in a process known as follicular activation, and the vast majority (99.9%) can become atretic before ovulation<sup>(5)</sup>. Despite the high rate of atresia that occurs in primordial follicles during *in vivo* growth and maturation, these can be a valuable source for studies about the *in vitro* development of oocytes, and the production of embryos<sup>(6,7)</sup>. Until recently, little was known about how the primordial follicles are activated and stimulated to grow and develop. In domestic mammals, early follicular development follows a very rigorous and complex process<sup>(8)</sup>; therefore, a long period of culture may be required for the development of preantral follicles. However, few studies of long-term *in vitro* culture have obtained follicular activation rates (sheep<sup>(9)</sup>, humans<sup>(10)</sup>, goat<sup>(11)</sup>). This may be related to the use of simple culture medium, without the addition of a series of growth factors or hormones that can satisfy the demands required by the follicles during the different stages of growth.

Among the most commonly used hormones in the culture medium, the follicle stimulating Hormone (FSH) outstands for its role in early folliculogenesis in mammals. FSH is a gonadotropin known for regulating the proliferation and differentiation of granulosa cells during the beginning (antral) of *in vivo* follicle development<sup>(12, 13)</sup>. In goats, the addition of 50 ng/mL of FSH to the culture medium of preantral follicles maintained the survival, and ultrastructural integrity of the follicles<sup>(14)</sup>.

The thyroxine hormone (T<sub>4</sub>) has also been implicated for its participation in the fertility of

mammals<sup>(15)</sup>. The effects of these hormones on ovarian folliculogenesis and steroidogenesis are related to the differentiation of granulosa cells<sup>(16)</sup>, and stimulation of ovulation<sup>(17)</sup>. Research has confirmed the addition of FSH and T<sub>3</sub> to the *in vitro* culture medium promotes the growth of preantral follicles, and that this interaction is mediated through the increase in the expression of mRNA to FSH receptors (rFSH) through a GDF-9 dependent mechanism. It is believed that these two hormones (FSH and T<sub>3</sub>) interact synergistically inhibiting the apoptosis of granulosa cells in small preantral follicles<sup>(18)</sup>.

The present study was designed with the aim to investigate the influence of human FSH (10 ng/mL) and T<sub>4</sub> (20 ng/mL) on the survival, activation, and growth of preantral follicles cultured *in vitro* for a long duration period (28 days). These concentrations were selected considering previous studies conducted by our team, which showed promising results with the use of these hormones.

## Material and Methods

This study was approved by the Ethics Committee on Animal Use, CEUA-UFV under case No 45/2012 on August 06, 2012. Ovaries (n = 10) from five adult non-pregnant mixed-breed goats with 1 to 3 y.o. were collected from a local slaughterhouse. Immediately postmortem, the ovaries were washed in 70% alcohol for 10 seconds and then washed again in MEM supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The pairs of ovaries were transported to the laboratory in MEM at 4 °C within 1 h postmortem. Unless otherwise mentioned, human FSH (hFSH), thyroxine and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, USA).

The organ culture system utilized herein was previously described<sup>(14, 19)</sup>. In the laboratory, the ovaries from each animal were stripped of surrounding fat tissue and ligaments. Subsequently, ovarian cortex tissue samples from each ovarian pair were cut into nine slices (approximate size: 3 x 3 mm, with 1 mm thickness) using a scalpel under sterile conditions. The tissue pieces were then either fixed for histological and ultrastructural analysis (fresh control) or placed in culture for 1 (d1), 7 (d7), 14 (d14), 21 (d21) and 28 (d28) days. Caprine tissues were transferred to 24-well culture dishes containing 1 mL of culture media. Culture was performed at 39 °C in 5% CO<sub>2</sub> in a humidified incubator and all the media were incubated for 1 h prior to use. The basic culture medium consisted of  $\alpha$ -MEM (pH 7.2 to 7.4) supplemented with 10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium (ITS), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin (BSA), which was called  $\alpha$ -MEM<sup>+</sup> (culture control).

The ovarian cortex fragments were cultured in  $\alpha$ -MEM<sup>+</sup> alone (culture control) and  $\alpha$ -MEM<sup>+</sup> supplemented with hFSH (10 ng/mL) in association with T<sub>4</sub> (20 ng/mL) as showed in the Table 1. Each treatment was repeated five times and the culture medium was changed every 2 days.

Before (fresh control) and after 1, 7, 14, 21 or 28 days in culture, all tissue pieces were fixed in Carnoy's solution for four hours, and then dehydrated in increasing concentrations of ethanol. After paraffin embedding (Vetec, Rio de Janeiro, Brazil), caprine ovarian cortex tissues were cut into 7-µm sections, which were mounted on glass slides and stained by Periodic Acid Schiff (PAS) - hematoxylin. Follicle stage and morphology were assessed microscopically on serial sections. Coded anonymized slides were examined by a microscope (Olympus, Japan) under 400x magnification.

The developmental stages of follicles have been defined previously<sup>(16)</sup> as primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened and cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells; and secondary: two or more layers of cuboidal granulosa cells around the oocyte). Only the preantral follicles (primordial, intermediate, primary and secondary) were considered in the analysis, and the antral stage was not included because of the very small number of antral follicles present in the ovarian fragments. The preantral follicles were also classified individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells that were well organized in one or more layers and lacked a pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. Overall, 150 follicles were evaluated for each treatment (30 follicles per treatment in one repetition x five repetitions = 150 follicles).

**Table 1:** Different means tested during *in vitro* culture of long duration

Culture medium	Abbreviation
$\alpha$ -MEM <sup>+</sup> (culture control for 1, 7, 14, 21 or 28 days)	$\alpha$ -MEM <sup>+</sup>
$\alpha$ -MEM <sup>+</sup> (T <sub>4</sub> + hFSH for 1, 7, 14, 21 or 28 days)	T <sub>4</sub> /hFSH

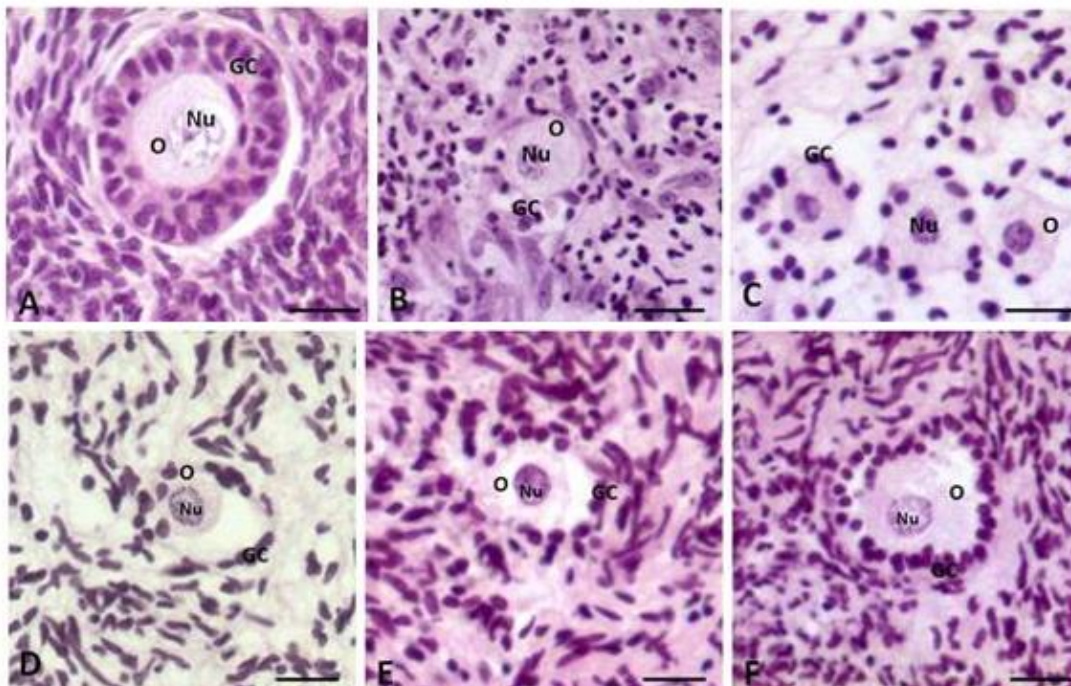
To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before (fresh control) and after culture in each medium. In addition, follicle and oocyte diameters were measured in healthy follicles only. Follicle diameter was recorded from edge to edge of the outer layer of granulosa cells or from the outside edge of the theca cell layer when present. Oocyte diameter was recorded from edge to edge of the oocyte membrane. Two perpendicular diameters were recorded in each measurement and the average of these two values was calculated. Each follicle was examined in every section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once.

To better evaluate follicular morphology the ultrastructural analysis was performed on preantral follicles from non-cultured tissue (fresh control), follicles cultured in the treatments that had the best results for morphology, activation and growth. Briefly, small pieces (1 mm<sup>3</sup>) of caprine ovarian tissues were fixed for 4 h at room temperature in karnovsky fixative (2.5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.2). After fixation, the samples were washed twice in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon 812 resin. Afterwards, semi-thin sections (3  $\mu$ m) were cut using an ultramicrotome (MT2-B, Sorvall, USA) stained with toluidine blue, and analyzed by light microscopy under 400x magnification. Ultra-thin sections (60 to 70 nm) were obtained from preantral follicles classified as morphologically normal in semi-thin sections, according to the criteria adopted for histological analysis. Subsequently, ultra-thin sections were contrasted with uranyl acetate, and lead citrate. A transmission electron microscope EM 109 Zeiss (EM, Berlin, Germany) operating at 80 Kv<sup>20</sup> was used to examine each sample. Parameters such as density and integrity of ooplasmic and granulosa cell organelles, vacuolization and integrity of the basement membrane were evaluated.

The variables were submitted to Normality (Lilliefors) and Homoscedasticity (Cochran) tests, and to the analysis of variance at a probability of 5%. In case significance was presented, Tukey test was carried out (SAS Institute Inc., Cary, NC, USA).

## Results

A total of 1,650 preantral follicles were analyzed by classic histology. Figures 1A, B, and C show normal follicles from the fresh control, and after the culture in  $\alpha$ -MEM<sup>+</sup> supplemented with T<sub>4</sub>/hFSH (day seven) or T<sub>4</sub>/hFSH (day 14), respectively. Figures 1D, E and F illustrate degenerated follicles after the culture in  $\alpha$ -MEM<sup>+</sup> (cultured control, day 28) or in media containing T<sub>4</sub>/hFSH (day 21) or T<sub>4</sub>/hFSH (day 28), respectively. In degenerated follicles, retracted oocytes and disorganization of granulosa cells were observed.



**Figure 1:** Histological sections of ovarian fragments tissue after staining Periodic Acid-Schiff's hematoxylin showing morphologically normal follicles (A, fresh control), (B, T<sub>4</sub>/hFSH - D7) and (C, T<sub>4</sub>/hFSH - D14). The degenerate follicles were represented by letters (D,  $\alpha$ -MEM<sup>+</sup> - D28); (E, T<sub>4</sub>/hFSH - D21) and (F, T<sub>4</sub>/hFSH - D28). O: oocyte; Nu: nucleus oocyte; GC: granulosa cells (Original magnification 200 $\times$ ). Scale bars represent 20  $\mu$ m.

The percentage of morphologically normal preantral follicles in the fresh control and after 1, 7, 14, 21 or 28 days of *in vitro* culture are shown in Table 2. In relation to the progression of culture from one to 28 days, there was a reduction ( $P < 0.05$ ) in the percentage of morphologically normal follicles in almost all treatments, compared to the fresh control (97.3%). However, with seven days of culture, the medium supplemented with T<sub>4</sub>/hFSH (20/10ng/mL,  $P > 0.05$ ) showed a rate of normal follicles similar to the non-cultured control.

When the  $\alpha$ -MEM<sup>+</sup> medium (cultured control) and the medium containing T<sub>4</sub>/hFSH were compared among themselves, for one to 28 days of culture, there was no difference ( $P > 0.05$ ) in the percentage of normal follicles. With the progression of the culture from one to 28 days, all media were able to maintain ( $P > 0.05$ ) the percentage of normal follicles during 21 days. However, the rate of normal follicles reduced when extending the culture for 28 days ( $P < 0.05$ ).

**Table 2:** Percentage (mean  $\pm$  SEM) of morphologically normal preantral follicles in fresh control (non-cultured) and after *in vitro* culture for 1, 7, 14, 21 or 28 days in the absence or presence of T<sub>4</sub>/hFSH (concentrations in ng/mL)

Treatments						
Control	97,3 $\pm$ 1,1					
	Day 1	Day 7	Day 14	Day 21	Day 28	
$\alpha$ -MEM <sup>+</sup>	83.3 $\pm$ 2.0 <sup>*aA</sup>	82.7 $\pm$ 1.9 <sup>*bA</sup>	78.0 $\pm$ 2.6 <sup>*aAB</sup>	80.7 $\pm$ 1.6 <sup>*aA</sup>	66.7 $\pm$ 4.6 <sup>*aB</sup>	
T <sub>4</sub> (20)/hFSH(10)	90.0 $\pm$ 0.0 <sup>*aA</sup>	90.7 $\pm$ 1.6 <sup>bA</sup>	80.7 $\pm$ 4.5 <sup>*aAB</sup>	72.7 $\pm$ 2.6 <sup>*aAB</sup>	66.0 $\pm$ 6.2 <sup>*aB</sup>	

\*Differs significantly from control follicles (P<0.05).

<sup>a,b</sup> differs significantly among concentrations in each day of culture (P<0.05).

<sup>A,B</sup> differs significantly with the progression of the culture period from days 1 to 28 in the same treatment (P<0.05).

The percentages of primordial and developing follicles in fresh ovarian tissue were 71.3% and 31.3%, respectively (Table 3). After 1, 7, 14, 21 and 28 days of culture, there was a reduction in this rate (P<0.05) in all media used, except in the one that contained T<sub>4</sub>/hFSH (20/10ng/mL, P>0.05) on day 1 of culture compared to the non-cultured control in primordial follicles. However, the rates of developing follicles of all media were similar to the non-cultured control (P>0.05), regardless of the time of culture. When the media were compared among themselves, no difference (P>0.05) was observed in both rates of primordial and developing follicles. With the progression of the culture period from one to 28 days, no differences in the percentages of primordial and developing follicles were observed (P>0.05).

**Table 3:** Percentage (mean  $\pm$  SEM) of primordial follicles and developing (intermediate, primary and secondary) in control (non-cultured) and after *in vitro* culture for 1, 7, 14, 21 or 28 days in the absence or presence of T<sub>4</sub>/hFSH (concentrations in ng/mL)

Primordial follicles						
Treatments						
Control	71.3 $\pm$ 4.2					
	Day 1	Day 7	Day 14	Day 21	Day 28	
$\alpha$ -MEM <sup>+</sup>	42.7 $\pm$ 5.9 <sup>*aA</sup>	34.7 $\pm$ 3.9 <sup>*aA</sup>	42.0 $\pm$ 3.9 <sup>*aA</sup>	32.0 $\pm$ 2.6 <sup>*aA</sup>	38.0 $\pm$ 3.3 <sup>*aA</sup>	
T <sub>4</sub> (20)/hFSH(10)	48.7 $\pm$ 6.1 <sup>aA</sup>	38.0 $\pm$ 8.1 <sup>*aA</sup>	44.7 $\pm$ 8.4 <sup>*aA</sup>	38.0 $\pm$ 4.4 <sup>*aA</sup>	33.3 $\pm$ 7.9 <sup>*aA</sup>	

Developing follicles						
Treatments						
Control	31.3 $\pm$ 7.4					
	Day 1	Day 7	Day 14	Day 21	Day 28	
$\alpha$ -MEM <sup>+</sup>	40.7 $\pm$ 7.1 <sup>aA</sup>	48.0 $\pm$ 3.8 <sup>aA</sup>	36.0 $\pm$ 4.7 <sup>aA</sup>	48.7 $\pm$ 3.7 <sup>aA</sup>	28.7 $\pm$ 3.2 <sup>aA</sup>	
T <sub>4</sub> (20)/hFSH(10)	41.3 $\pm$ 6.1 <sup>aA</sup>	52.7 $\pm$ 9.4 <sup>aA</sup>	36.0 $\pm$ 8.1 <sup>aA</sup>	34.7 $\pm$ 3.3 <sup>aA</sup>	32.7 $\pm$ 8.6 <sup>aA</sup>	

\*Differs from control follicles (P<0.05).

<sup>a,b</sup> differs among concentrations in each day of culture (P<0.05).

<sup>A,B</sup> differs with the progression of the culture period from days 1 to 28 in the same treatment (P<0.05).

There was a reduction in the oocyte diameter (P<0.05) throughout the culture in all media, except when were cultured for one day with  $\alpha$ -MEM+ and T<sub>4</sub>/hFSH (Table 4). However, the  $\alpha$ -MEM+ with day one of culture and the T<sub>4</sub>/hFSH cultured for one and seven days maintained follicular diameter (P>0.05) when compared to the non-cultured control. When the media were compared among themselves, there was no difference (P>0.05) in oocyte and follicle diameters. With the



progression of culture from one to seven days, the  $\alpha$ -MEM+ and the media T<sub>4</sub>/hFSH maintained oocyte and follicular diameter ( $P>0.05$ ), occurring later a reduction of both, when extending the culture up to 28 days.

**Table 4:** Caprine oocyte and follicle diameters (mean  $\pm$  SEM) in non-cultured tissues (fresh control) and in tissues cultured for 1, 7, 14, 21 or 28 days in the absence or presence of interaction T<sub>4</sub>/hFSH (concentrations in ng/mL)

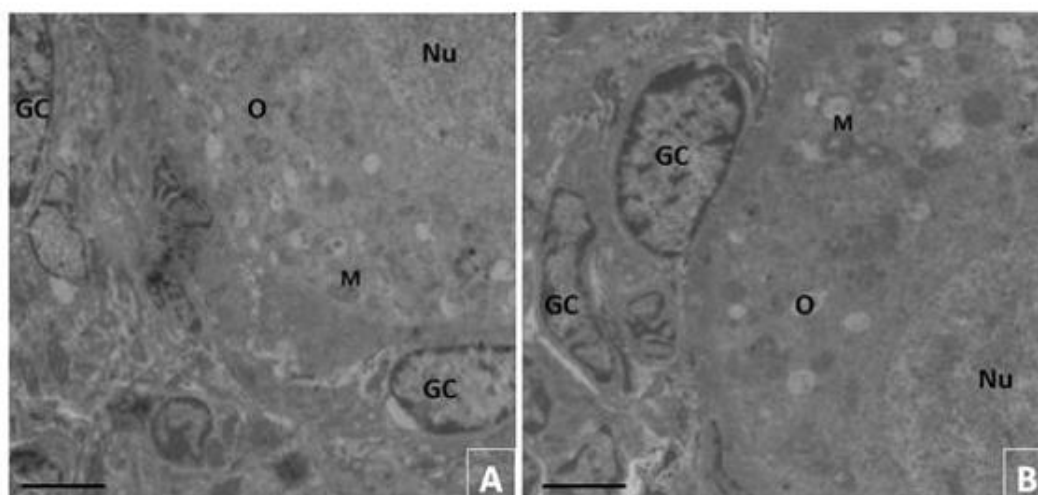
		Oocyte diameter ( $\mu\text{m}$ )				
Treatments		Day 1	Day 7	Day 14	Day 21	Day 28
Control		26.0 $\pm$ 0.6				
$\alpha$ -MEM+		24.2 $\pm$ 0.8 <sup>aA</sup>	22.7 $\pm$ 0.7 <sup>aAB</sup>	21.2 $\pm$ 0.6 <sup>aB</sup>	20.8 $\pm$ 1.0 <sup>aB</sup>	20.3 $\pm$ 0.5 <sup>aB</sup>
T <sub>4</sub> (20)/hFSH(10)		24.7 $\pm$ 0.8 <sup>aA</sup>	23.7 $\pm$ 0.6 <sup>aAB</sup>	20.7 $\pm$ 0.8 <sup>aBC</sup>	21.7 $\pm$ 0.9 <sup>aBC</sup>	19.6 $\pm$ 0.6 <sup>aC</sup>
		Follicle diameter ( $\mu\text{m}$ )				
Treatments		Day 1	Day 7	Day 14	Day 21	Day 28
Control		33.4 $\pm$ 0.8				
$\alpha$ -MEM+		32.5 $\pm$ 1.2 <sup>aA</sup>	30.2 $\pm$ 1.2 <sup>aAB</sup>	27.8 $\pm$ 0.7 <sup>aB</sup>	27.8 $\pm$ 1.3 <sup>aB</sup>	27.6 $\pm$ 0.7 <sup>aB</sup>
T <sub>4</sub> (20)/hFSH(10)		33.2 $\pm$ 1.0 <sup>aA</sup>	30.7 $\pm$ 0.7 <sup>aAB</sup>	27.5 $\pm$ 0.9 <sup>aBC</sup>	29.0 $\pm$ 1.1 <sup>aB</sup>	25.5 $\pm$ 0.6 <sup>aC</sup>

\*Differs significantly from control follicles ( $P<0.05$ ).

<sup>a,b</sup>differs significantly among concentrations in each day of culture ( $P<0.05$ ).

<sup>A,B</sup>differs significantly with the progression of the culture period from days 1 to 28 in the same treatment ( $P<0.05$ ).

The ultrastructural analysis was performed in preantral follicles of the non-cultured control and of follicles cultured for one and seven days in medium with T<sub>4</sub>/hFSH (20/10ng/mL). This medium with hormones was selected for presenting satisfactory results on the parameters evaluated by the histological analysis.



**Figure 2:** Ultrastructural analysis of preantral follicles from fresh control (A, 3.000x) cultured for seven days (B, 4.400x) in medium containing T<sub>4</sub>/hFSH (20/10ng/mL). Note homogeneous cytoplasm, and mitochondria rounded characteristic of the fresh control follicles (A and B). O: oocyte; Nu: oocyte nucleus; GC: granulosa cells; M: mitochondria. (A and B = 5  $\mu\text{m}$ ).

The follicles of the non-cultured control revealed an intact nuclear envelope with a large oocyte nucleus. Moreover, the organelles, particularly the mitochondria and endoplasmic reticulum, were

uniformly distributed in the cytoplasm. The granulosa cells were well organized around the oocyte (Fig. 2A). When cultured for seven days in medium with T<sub>4</sub>/hFSH (20/10ng/mL, Fig 2B), the ultrastructural characteristics of the follicles were similar to the non-cultured control. After that culture, the oocytes showed ultrastructural normality, basal membranes, nuclear envelope, as well as in the large oocyte nucleus (Fig. 2A and 2B, respectively). In addition, the follicles exhibited uniformly distributed organelles in the cytoplasm, particularly the endoplasmic reticulum and the mitochondria. The granulosa cells were normal and well organized around the oocyte, presenting also a large, and elongated nucleus (Fig. 3B).

## Discussion

The addition of hormones and growth factors is essential to the success of *in vitro* culture of preantral follicles (PAF) of domestic animals as demonstrated in the literature<sup>(21-23)</sup>. In this context, the development of an *in vitro* culture system is fundamental for the achievement of a greater number of oocytes included in preantral follicles<sup>(21)</sup> therefore, a better comprehension of the different substances that regulate follicular and oocyte development is fundamentally important for the success of *in vitro* culture systems, with the final objective of producing a greater number of viable embryos.

There are several hormones and growth factors tested in experiments at the present time. It is well known that FSH is outstanding for carrying out an important participation in early folliculogenesis in mammals, in addition to stimulating the survival growth and antrum formation in *in vitro* culture systems<sup>(24)</sup>. Thyroid hormones T<sub>3</sub> and T<sub>4</sub> demonstrated the capacity to influence cell differentiation and the formation of the follicular antrum in PAF submitted to *in vitro* culture<sup>(25)</sup>. The association of thyroid hormones with FSH was demonstrated to be effective in *in vitro* development of preantral follicles of rats<sup>(26)</sup>, and in antrum formation, and ovulation of sheep PAF cultured *in vitro*<sup>(2)</sup>. This association (hFSH + T<sub>4</sub>) was also beneficial in the present study because it showed a satisfactory effect by keeping the oocyte diameter after 1 day of culture and promoting the activation of primordial follicles on days 1, 7, 14, 21 or 28 of culture.

When analyzing the survival rate of the cultured preantral follicles in the present work, we noted that the group cultured in the medium T<sub>4</sub>/hFSH practically maintained the survival rate of the follicles when compared to the  $\alpha$ -MEM<sup>+</sup> medium without supplementation during the culture for one, seven, 14, 21 or 28 days. These data corroborated those found by Alves<sup>(27)</sup>, who, using a similar culture medium, maintained the follicular survival for a period of eight days. Apparently,  $\alpha$ -MEM<sup>+</sup> allows follicular survival considering that it is a compound of amino acids and vitamins, essential for follicular development<sup>(28, 11)</sup>. Additionally, the enrichment of this medium with the hormones T<sub>4</sub> and hFSH may contribute even more to the follicular development and survival, due to the direct effect of these in either the stimulation of granulosa cells and growth factors as in the proliferation of FSH and LH receptors and in follicular maturation<sup>(15, 29)</sup>.

During day 7 (D7) of culture, it was possible to observe that T<sub>4</sub>/hFSH medium was also able to maintain the viability rate of the follicles when compared to the non-cultured control. This situation can be explained due to the latency period of T<sub>4</sub> hormone, as it is believed it stays in a latency period of two to three days after its administration, and its action reaches its maximum peak after 10 days of culture<sup>(25)</sup>. Therefore, due to the delay in the activation of this hormone, the positive effect



of the association T<sub>4</sub>/hFSH can be noted only on the 7<sup>th</sup> day of culture.

However, comparing the culture progression of the media used in our study, the percentage of normal follicles significantly reduced ( $P < 0.05$ ) from D21 to D28, and from D7 to D28 days, in the  $\alpha$ -MEM<sup>+</sup> and T<sub>4</sub>/hFSH media, respectively. It should be emphasized that the use of too long culture period has some survival growth limitations, and differentiation of preantral follicles due to the prolonged culture period. However, it has been found that the addition of substances to the medium can contribute to the improvement of the survival rates after the culture. It is believed that, during long culture periods of preantral follicles, the addition of antioxidant substances to the culture medium, such as ascorbic acid, is essential to maintain cellular integrity<sup>(11)</sup>. This author also reports that the combination of FSH and ascorbic acid is able to promote a synergetic effect providing a significant increase in oocyte and follicular diameter after seven days of culture. Therefore, the reduction of viable follicles from day 21 to 28 of culture observed in our work could be avoided by adding some substance with a similar effect to the one described.

Moreover, as found in other studies, the low concentration of hFSH used in the present study may have made the follicular survival unviable during a prolonged period of culture. This fact could be previously verified by other researchers, as Silva et al.<sup>(16)</sup>, who found no significant effect of FSH on the survival of follicles after 5 days of culture, possibly due to a high concentration of FSH (100ng/mL) used. Conversely, Matos et al.<sup>(14)</sup> maintained follicular survival after seven days of culture with the addition of 50ng/mL of FSH to the culture medium. Therefore, we can evaluate that the concentration of hFSH used in this study was sufficient to maintain the survival until the seventh day of culture; however, it is suggested that after this period this concentration was insufficient, because it was not able to maintain follicular survival in the long duration culture.

Different results obtained using FSH in the culture of preantral follicles can be justified due, especially, to its origin and purity level. Despite the use of FSH from the pituitary extract of domestic species such as pigs (pFSH) and sheep (oFSH), as well as of humans (hFSH) in several studies, it is believed the recombinant FSH (rFSH) produced from the recombinant DNA technique is the purest and most homogeneous form<sup>(30)</sup>; however, its high cost makes its use in scientific research unviable. Due to the use of hFSH in the present work, the results achieved in the long duration cultures may have been damaged, mainly by its purity level and heterogeneity, that directly interfere in cultures for prolonged periods.

The follicular activation is measured by the ability of differentiation of granulosa cells, resulting in the transition of primordial follicles to intermediate, primary, and secondary follicles<sup>(31)</sup>; however, the knowledge about the factors and mechanisms involved in the activation of primordial follicles is scarce, mainly because of the difficulty of isolating these follicles<sup>(32)</sup>. The evidence of follicular activation in preantral follicles cultured *in vitro* is noted for the reduction of the number of primordial follicles, and consequent increase of developing follicles along the days of culture<sup>(33, 3)</sup>. In all media tested in this study ( $\alpha$ -MEM<sup>+</sup> and T<sub>4</sub>/hFSH), there was activation of primordial follicles on days 1, 7, 14, 21 or 28 of culture, except on day 1 of the T<sub>4</sub>/hFSH medium. This fact can be observed by the reduction of primordial follicles compared to the control; nevertheless, a concomitant increase in developing follicles throughout the days of culture that was expected did not occur, since in all treatment the rates of developing follicles were similar to the non-cultured control. These findings disagree with the ones found by Matos et al.<sup>(14)</sup>, who worked with the same concentration of FSH (10 ng/mL), and observed there was not a reduction of primordial follicles and concomitant increase of developing follicles on day 1 of culture; however, at higher

concentrations (50 and 100 ng/mL), this authors obtained similar results as ours. The source of FSH used, however, differed between studies, in the work in question porcine FSH was used, different from the one used in our work, human FSH.

In the present study, despite the reduction of primordial follicles during all days of culture, the increase in the developing follicles was not observed on any day of culture, in any researched media; thus, none of the tested media were able to sustain follicular activation initiated by them in primordial follicles on days 1 ( $\alpha$ -MEM<sup>+</sup> cultured control), 7, 14, 21 or 28 (T<sub>4</sub>/hFSH and  $\alpha$ -MEM<sup>+</sup>) of culture, mainly by the similarity between the rates of developing follicles to the non-cultured control and the media tested during all days of culture. These results contradict the ones by Matos et al.<sup>(14)</sup>, in which, after seven days of culture, follicles maintained their development. Combining our data with follicular survival, herein presented, we suggest that the development of the follicles in question was not continuous after the 1<sup>st</sup> day of culture due to the rise of their mortality rate.

Despite the considerable anti-apoptotic effect of gonadotropic hormones (LH and FSH<sup>(34)</sup>) mediated by the action of IGF-I<sup>(35)</sup>, in our study, it was not possible to observe their activation and development after day 1 of culture, reflecting in high mortality rates in the tested media, and similarity between the developing follicles to the non-cultured control and  $\alpha$ -MEM<sup>+</sup> and T<sub>4</sub>/hFSH media. This may have occurred due to the gaseous atmosphere of the culture kiln by not providing a suitable environment for the culture of the follicles. Furthermore, it is possible the proposed media were not totally sufficient to supplement and sustain the development of the follicles, since the growth factors and hormones are fundamental for the development of the follicle in each stage of its development and occasionally small differences of concentrations or synergism between the two hormones can negatively contribute to follicular survival.

In relation to the diameter of the oocyte and the follicle, none of the tested media were capable of maintaining the growth of the follicles through the culture, except for  $\alpha$ -MEM<sup>+</sup> on day 1 of culture, and T<sub>4</sub>/hFSH on days 1 and 7 of culture. In contrast, studies have already proved the addition of FSH to the culture medium was able to promote the increase of preantral follicles cultured *in vitro*<sup>(36, 14, 2)</sup>. In the presence of 10 and 50 ng/mL of FSH, Matos et al.<sup>(14)</sup> observed an increase in the diameter of both the oocyte and the caprine follicle on day 7 of culture. Itoh et al.<sup>(36)</sup> also demonstrated that 50ng/mL of FSH increased both the diameter of the oocyte, and of bovine follicles cultured for 13 days. These authors are based on the concept of the existence of FSH receptors during the progressive development of PAF, in both the transition from primordial to primary follicles, and to secondary follicles<sup>(37)</sup>; therefore, it is possible that FSH acts on both cell types to promote growth, and follicular development<sup>(38)</sup>.

In agreement with these authors, Arunakumari et al.<sup>(2)</sup> reported an increase in the diameter in sheep PAF but using other growth inducers as IGF-I and GH and also the combination of FSH + T<sub>4</sub>. However, it is not always that the T<sub>4</sub>/FSH association can be considered capable of stimulating the development of caprine PAF. Wongbandue et al.<sup>(39)</sup> also did not achieve satisfactory results with the addition of T<sub>4</sub>, but they used the hormone at the concentrations of 0.5, 1.0 and 2.0  $\mu$ g/mL added in base culture medium M199 supplemented with 2.13  $\mu$ g/mL of FSH, besides growth factors and antibiotics. At none of these concentrations there was a difference or gain in the diameter in feline follicles cultured for 0, 3, 7 and 14 days. This study agrees with ours, and the most coherent explanation is that the expression of T<sub>4</sub> receptors in granulosa cells possibly develops in stages of follicular development, different from what was used in ours study. Although follicular diameter did not increase in our work through the culture, it did not present a reduction on days 1 and 7 of

culture of T<sub>4</sub>/hFSH medium, if it happened indeed it could indicate a process of atresia. Thus, the observation of the similarity of the diameter in relation to the non-cultured control group greatly showed the viability of the medium tested in the initial period of culture.

Ultimately, we used TEM for a better ultrastructural evaluation of the follicles quality. This tool has been used in several studies to prove the existence of possible ultrastructural damage caused by culture systems that may not be morphologically perceptible<sup>(40, 41, 42)</sup>. In the present study, the ultrastructural features of the follicles cultured in the presence of hFSH and T<sub>4</sub> for seven days were maintained similar to the non-cultured control. The cellular structures, such as endoplasmic reticulum and mitochondria, were evenly distributed in the cytoplasm and the granulosa cells were ultrastructurally normal, and well organized around the oocyte. Other studies also demonstrate that FSH maintains the ultrastructure of caprine PAF after the *in vitro* culture for seven days<sup>(14)</sup>. Matos et al.<sup>(14)</sup> also verified that the ultrastructural integrity of the follicles was maintained after 1 day of culture in  $\alpha$ -MEM<sup>+</sup> medium, and after 7 days in  $\alpha$ -MEM<sup>+</sup> + FSH (50ng/mL). On the other hand, Rossetto et al.<sup>(11)</sup> demonstrated the integrity of follicles cultured after 14 days in media containing ascorbic acid and FSH.

In the case of a culture for a prolonged time, the ultrastructural integrity of PAF was also observed by Gutierrez et al.<sup>(43)</sup>, who evaluated culture media for bovine PAF with FSH, EGF, and IGF-I for 28 days. In this system, most follicles maintained their morphology throughout the culture, in the presence of a layer of theca, and basement membrane surrounding the granulosa cells. Moreover, these authors also observed antrum formation, which occurred between the days 10 and 28 of culture. However, because our study was conducted with other species, the chronological comparison of these events is not possible.

Given the previously presented and discussed results, we noted that, although the medium tested in this experiment T<sub>4(20)</sub>/hFSH<sub>(10)</sub> has not provided a substantial growth in the follicular and oocyte diameter, it did not impair the activation and viability of caprine preantral follicles cultured *in vitro* for a period of seven days. The synergistic effect between T<sub>4</sub> and FSH can be explained by the action of thyroid hormones in the amplification of FSH effect on the differentiation of granulosa cells, fact demonstrated by Maruo et al.<sup>(44)</sup> and experimentally proved later by Arunakumari et al.<sup>(2)</sup>. The action of thyroid hormones on the expression of mRNA of FSH receptors in granulosa cells is still very controversial and needs to be studied and defined more precisely.

## Conclusion

The medium tested in this experiment T<sub>4(20)</sub>/hFSH<sub>(10)</sub> is able to maintain the survival, follicular activation, and ultrastructural integrity of preantral follicles of goat cultured *in vitro* for a period of up to seven days, which validates and enables the use of the medium tested in culture systems for this species.

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